A comparative study of the efficiency of a probiotic and the anti-K99 and anti-A14 vaccines in the control of diarrhea in calves in Brazil

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A total of 99 pregnant cows were divided into eight groups submitted to the following treatments : group I (n= 29) consisted of unvaccinated cows whose calves did not receive a probiotic and was used as control. Group II (n = 10) consisted of vaccinated cows whose calves did not receive a probiotic. Groups III, IV and V (n = 10 neach) consisted of vaccinated cows whose calves received a probiotic for 5, 15 and 30 days, respectively. Groups VI, VII and VIII (n = 10 each) consisted of unvaccinated cows whose calves received a probiotic for 5, 15 and 30 days, respectively. Each animal in the vaccinated groups received two 5.0 ml vaccine doses containing pili K99 and A14 of *Escherichia coli* by the subcutaneous route. The probiotic containing *Lactobacillus acidophilus* at the dose of 2.0 × 10^8live cells in 250 ml milk, was administered orally. All animals were observed clinically and bacteriologically and anti-K99 and anti-A14 antibody titers were determined in serum and colostrum. Mean calf weight was measured at birth and at 30 days of age. The results showed that a combination of the vaccine with the probiotic administered for 15 and 30 days was the most efficient treatment for the control of diarrhea.

Key words : Cattle - Calf - Diarrhoea - *Escherichia coli* - Probiotics - Vaccine - Disease control - Brazil.

INTRODUCTION

Disorders of the digestive tracts have been frequently reported to occur among calves during the first weeks of life. Among the different causes of diarrhea in calves, enterotoxigenic *Escherichia coli* is the most common. The development of diarrhea due to enterotoxigenic *Escherichia coli* depends on two factors : colonization of the small intestine and production of enterotoxin (9).

Pili K99 (11) and A14 (6) are filamentous appendages of protein constitution that are produced by some bacteria and play an important role in *Escherichia coli* colonization of the small intestine of calves. The ingestion of colostrum from cows immunized against K99-carrying *Escherichia coli* has been reported to be efficient for the protection of calves against colibacillosis (3). However, the appearance of new adherence antigens (5) has motivated the development of new vaccines potentiated with these antigens.

An important characteristic of the good functioning of the intestinal tract is the equilibrium of its bacterial microflora. This equilibrium is very important but quite difficult to determine. It is mainly harmful to pathogenic microorganisms which are able to multiply rapidly and which often pre-exist in the intestinal flora. This equilibrium may be altered by aggressive phenomena that modify the intestinal secretions and peristalsis (10, 13). In these cases, pathogens such as *Escherichia coli* present in a state of latency may proliferate.

Preparations of live microorganisms such as *Lactobacillus*, called probiotics, are commonly added as a dietary supplement to maintain and stabilize a population of beneficial organisms in the gastrointestinal tract, thus improving cattle growth and the efficiency of food absorption in these animals (12). Thus, the role of a probiotic as a microbial bioregulator is to maintain the equilibrium of the intestinal flora, with the major function of preventing intestinal colonization by pathogenic bacteria such as enterotoxigenic *Escherichia coli* (4).

The objective of the study was to compare the efficiency of a probiotic, vaccination and a combination of the two in the control of enterotoxigenic *Escherichia coli*-induced diarrhea in calves.

MATERIALS and METHODS

Probiotic

The probiotic used was a *Lactobacillus acidophilus* (LBV-1) strain isolated from the gastrointestinal tract of an adult cow by the method of Wolf *et al.* (14) with the following characteristics : apathogenicity, lactic acid production and resistance to pH between 4.0 and 3.0.

Probiotic dose

The inoculum contained 2.0 × 10^8 live *Lactobacillus acidophilus* cells Tournut *et al.* (13) in lyophilized form in 10 g of an inert substance (kaolin) as vehicle.

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Probiotic inoculation

The probiotic was administered to each calf by the oral route mixed with 250 ml of milk starting on the first day of life.

Vaccine

Composition

The experimental vaccine was prepared with two *Escherichia coli* strains, $K99^+$ Sta^+ and $A14^+$ Sta^-. Cultures were inactivated with 40% commercial formalin and adsorbed to aluminum hydroxide. The vaccine dose was 5.0 ml and contained 5.0×10^9 bacteria of each *Escherichia coli* strain.

Protocol of vaccination

Each pregnant cow from the vaccinated groups received two vaccine doses of 5.0 ml by the subcutaneous route. The first dose was administered during the 5th or 6th week before parturition and the second three weeks after the first. The animals that were not vaccinated were similarly injected with a 5.0 ml dose of placebo (sterile saline) at the same times as the vaccinated animals.

Animals

The authors used 99 pregnant cows of Pitangueiras breed (milk breed) belonging to the « Três Barras » Farm, municipality of Pitangueiras, SP, Brazil. The animals were divided into eight groups submitted to the following treatments : group I (n = 29) consisted of unvaccinated cows whose calves did not receive a probiotic and was used as control. Group II (n = 10) consisted of vaccinated cows whose calves did not receive a probiotic. Groups III, IV and V (n = 10 each) consisted of vaccinated cows whose calves received a probiotic for 5, 15 and 30 days, respectively. Groups VI, VII and VIII (n = 10 each) consisted of unvaccinated cows whose calves received a probiotic for 5, 15 and 30 days, respectively.

Clinical observation

All passively immunized, probiotic-treated and control calves were examined clinically throughout the experiment for the occurrence of diarrhea and for weight gain. Fecal swabs were obtained from the animals with diarrhea and submitted to bacteriologic examination, and affected animals were treated immediately.

Bacteriology

Isolation and biochemical identification

Fecal samples were inoculated into MacConkey agar and incubated at 37-72° for 24 hours. Typical *Escherichia coli* colonies were identified by the following tests : lactose fermentation, indole production, methyl red and Voges & Proskauer reactions, citrate utilization, urease formation, and H_2S production. Readings were taken after 24 hours of incubation at 37 °C.

Detection of K99 and A14

Anti-K99 and anti-A14 sera were prepared by immunizing rabbits against purified pilus A14 and against strain K12 : K99 cultured at 37 °C, as described by Edwards and Ewing (8). The anti-K12 : K99 serum was absorbed with a homologous strain cultured at 18 °C. The *Escherichia coli* strains employed for the detection of K99 and A14 were cultured on Minca agar at 37 °C for 18 hours and examined by the agglutination test on a slide by the technique of Ávila *et al.* (5).

Detection of Sta enterotoxin

The *Escherichia coli* strains were cultured in brain-heart infusion broth (BHI) on a waterbath under shaking at 150-200 rpm, at 37o for 18 hours and then centrifuged. Evans Blue (2 %) was added to the supernatant of each strain and 0.1 ml of the mixture was inoculated by the intragastric route into 3 mice aged 3 to 4 days. Another mouse of the same age was similarly inoculated with 0.1 ml BHI broth and Evans Blue and maintained as a control according to the technique of Dean *et al.* (7).

Serology

Blood samples were collected from each cow before vaccination or placebo administration and on the day of parturition, when a colostrum sample was also obtained for the determination of the presence of anti-K99 and anti-A14 antibodies. Antibody titers were determined by the agglutination technique using antigens purified by the method of Acres (1). The titer is reported as the reciprocal of the highest serum dilution that showed agglutination.

The *Escherichia coli* strains were identified serologically using OK antisera produced in rabbits by repeated injections of live cultures against the following *Escherichia coli* strains : Myers 483 (O9 : K35 : K99), Myers 490 (O101 : K30 : K99), Myers 505 (O101 : K28 : K99), Myers 524 (O8 : K85 : K99), Myers 559 (O9 : K25 : K99), and Myers Wi-1 (O20 : K ? : K99). Strains were identified by the agglutination test on a slide according to the method of Ávila *et al.*(5).

Statistical analysis

The experiment was carried out using a fully randomized design with controls for each treatment group. The effects of the various treatments were evaluated by analysis of variance for the quantitative characteristics (weight gain), and by the chi-square test for the qualitative characteristic (diarrhea) (2).

RESULTS

Table I shows the number of animals (cows and calves) in each group and the respective numbers of isolations of *Escherichia coli* carrying or not the Sta enterotoxin.

Table II lists the agglutinating antibody titers against antigens K99 and A14 present in the serum and colostrum of the cows in each group. Of the 99 pregnant cows, 28 presented agglutinating titers for pili K99 and A14 in serum before the application of the first dose of vaccine or placebo. These titers ranged from 2 to 4.

The number and percentage of calves with diarrhea in each group are listed in Table III.

TABLE I

Number of calves born and strains of *Echerichia coli* K99⁺ Sta⁺ or A14⁺ Sta⁻ isolated from the feces of calves with diarrhea in each group

Groups	Number of cows	Number of calves born	No. of *E. coli* strains	
			K99⁺ Sta⁺	A14⁺ Sta⁻
I	29	29	15	05
II	10	10	03	—
III	10	10	02	01
IV	10	10	02	—
V	10	10	01	01
VI	10	10	03	01
VII	10	10	02	01
VIII	10	10	03	—

Table IV shows the mean weight data for the calves in each group at birth and at 30 days of age, as well as the mean weight gains.

TABLE II

Titers of agglutinating antibodies against K99 and A14 present in the serum and colostrum of the cows from each group

Groups	Number of cows	Serum titers against K99		Serum titers against A14		Titers in colostrum	
		before immunization	parturition	before immunization	parturition	K99	A14
I	29	*0-2	0-4	0-4	0-4	0-4	0-4
II	10	0-2	40-160	0	40-320	80-160	80-640
III	10	0-2	20-160	0-2	80-640	40-160	80-640
IV	10	0	40-160	0	40-320	40-160	80-1 280
V	10	0-2	40-160	0	80-320	80-160	80-640
VI	10	0	0	0	0	0	0
VII	10	0-2	0-4	0-2	0-2	0-4	0-2
VIII	10	0-2	0-2	0	0	0-4	0-2

* No titers.

TABLE III

Number and percentage of calves with diarrhea caused by *Escherichia coli* in each group

Groups	Number of calves born	Cases of calves with diarrhea	
		Number	%
I	29	20	68.9
II	10	03	30.0
III	10	03	30.0
IV	10	02	20.0
V	10	02	20.0
VI	10	04	40.0
VII	10	03	30.0
VIII	10	03	30.0

TABLE IV

Mean weights of the calves in each group at birth and at 30 days of age and respective weight gains

Groups	Number of calves	Mean weight (kg) per age (days)		Mean weight gain (kg)
		01 day	30 days	
I	29	32.3	38.8	6.5 a[1]
II	10	27.7	38.1	10.4 b
III	10	29.6	42.4	12.8 c
IV	10	28.7	43.7	15.0 d
V	10	29.3	43.8	14.5 d
VI	10	32.3	42.0	9.7 b
VII	10	29.1	41.6	12.5 c
VIII	10	30.7	41.2	10.5 c

1. Equal letters in the column not indicate significant difference ($p > 0,01$).

The percentage, number and serotypes of *Escherichia coli* isolated from the feces of calves with diarrhea in each group are listed in Table V.

TABLE V
Percentage, number and serotypes of *E. coli* isolated from the feces of calves with diarrhea in each group

Groups	Number of calves born	Percentage of isolation	Number of serotypes	Serotypes isolated
I	29	68.9	08	O8 : K85 : K99
			03	O8 : K25 : K99
			02	O8 : K35 : K99
			02	O101 : K30 : K99
			05	O ? : K ? : A14
II	10	30.0	02	O8 : K85 : K99
			01	O101 : K30 : K99
III	10	30.0	01	O9 : K35 : K99
			01	O8 : K25 : K99
			01	O ? : K ? : A14
IV	10	20.0	01	O8 : K85 : K99
			01	O101 : K30 : K99
V	10	20.0	01	O101 : K30 : K99
			01	O ? : K ? : A14
VI	10	40.0	02	O8 : K85 : K99
			01	O101 : K30 : K99
			01	O ? : K ? : A14
VII	10	30.0	02	O8 : K85 : K99
			01	O ? : K ? : A14
VIII	10	30.0	01	O8 : K25 : K99
			02	O101 : K30 : K99

DISCUSSION

On the basis of the results of the agglutination test on a slide against the two adherence antigens (Table I), it can be seen that of the *Escherichia coli* strains isolated from calves with diarrhea, 31 (31.3 %) produced pilus K99, and 9 (9.1 %) produced pilus A14. The Dean test showed that all $K99^+$ strains were Sta^+, whereas the $A14^+$ strains were Sta^-.

Table II presents the values of the agglutinating antibody titers against antigens K99 and A14 present in the serum and colostrum of all cows. Agglutinating titers ranging from 2 to 4 were detected in the serum of 24 cows before immunization or placebo for both pili. The antibody titers present in the blood sera of control cows and of unvaccinated cows whose calves received a probiotic presented incipient elevation in colostrum. No animal presented an agglutinating titer for the two antigens before immunization in group IV. Group II animals did not present antibodies against pilus A14 before vaccination. No animal in group VI presented an agglutinating titer in serum or colostrum against the two adherence antigens before the application of the first dose of placebo or at the time of parturition. Significant increases were detected in the serum titers of the cows after vaccination. These increases were also observed in antibody titers in the colostrum of vaccinated cows and especially against antigen A14. These facts agree with data reported by Ávila *et al.* (3) with respect to postvaccinal antibody transfer from serum to colostrum and from colostrum to the calves.

The largest percentage of calves with diarrhea was 68.9% and occurred in group I whose cows were not vaccinated and whose calves did not receive a probiotic. Statistical analysis by the chi-square test showed significant differences ($p < 0.05$) between this group (control) and the treated groups (Table III).

Analysis of variance showed significant differences ($p < 0.01$) between treatments with respect to weight gain (Table IV). The least significant difference was 2.7 kg and the treatments that proved to be most efficient were those for groups IV and V, whose cows were vaccinated and whose calves received a probiotic for 15 and 30 days, respectively. Group III whose cows were vaccinated and whose calves received a probiotic for 5 days and groups VII and VIII whose calves received a probiotic for 15 and 30 days, respectively, showed equivalent efficiency, although they were not as good as groups IV and V whose cows were vaccinated and whose calves received a probiotic for 15 and 30 days, respectively. Group II whose cows were vaccinated and whose calves received no probiotic, and group VI whose cows were not vaccinated and whose calves received a probiotic for 5 days were not as efficient as the previous groups.

Three serotypes bearing the K99 pilus were isolated and belonged to serogroups O8, O9 and O101 (Table V). The serotypes bearing pilus A14 were not classified. In animals that received a probiotic and that presented diarrhea, the presence of *Escherichia coli* in the feces was predominant and isolation of *Lactobacillus* was difficult. However, in the animals without diarrhea, the presence of *Lactobacillus* seemed to predominate in feces, facilitating the isolation of this bacterium. This observation has also been reported by Savage (10) and Tournut *et al.* (13). These facts seem to confirm the function of the probiotic reported by Tournut *et al.* (13), i.e., preventing intestinal colonization by enterotoxigenic *Escherichia coli* (4). However, more specific studies are needed, also including other animal species.

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ÁVILA (F.A.), PAULILLO (A.C.), SCHOCKEN-ITURRINO (R.P.), LUCAS (F.A.), ORGAZ (A.), QUINTANA (J.L.). Etude comparative de l'efficacité d'un probiotique et de vaccins anti-K99 et anti-A14 pour la prophylaxie de la diarrhée chez les veaux au Brésil. *Revue Élev. Méd. vét. Pays trop.*, 1995, **48** (3) : 239-243.

Quatre-vingt-dix-neuf vaches gestantes ont été réparties en huit groupes et soumises au traitement suivant : le groupe I (29 sujets) comprenait des vaches non vaccinées dont les veaux n'avaient pas reçu de probiotique et servait de témoin. Le groupe II (10 sujets) se composait de vaches vaccinées dont les veaux n'avaient pas reçu de probiotique. Les groupes III, IV et V (10 sujets chacun) se composaient de vaches vaccinées dont les veaux avaient reçu un probiotique pendant 5, 15 et 30 jours respectivement. Les groupes VI, VII et VIII (10 sujets chacun) étaient constitués de vaches non vaccinées dont les veaux avaient reçu un probiotique pendant 5, 15 et 30 jours respectivement. Chaque animal appartenant aux groupes vaccinés a reçu deux doses vaccinales de 5,0 ml contenant des pili K99 et A14 d'*Escherichia coli*, administrées par voie sous-cutanée. Le probiotique qui renfermait du *Lactobacillus acidophilus* à la dose de $2{,}0 \times 10^8$ cellules vivantes dans 250 ml de lait, a été administré par voie orale. Tous les animaux ont été soumis à une observation clinique et bactériologique, et les titres d'anticorps anti-K99 et anti-A14 ont été déterminés dans le sérum et dans le colostrum. Le poids moyen des veaux a été contrôlé à la naissance, puis à l'âge de trente jours. Les résultats montrent que la combinaison du vaccin avec le probiotique administré pendant 15 et 30 jours a constitué le traitement le plus efficace pour contrôler la diarrhée.

Mots-clés : Bovin - Veau - Diarrhée - *Escherichia coli* - Probiotique - Vaccin - Contrôle des maladies - Brésil.

ÁVILA (F.A.), PAULILLO (A.C.), SCHOCKEN-ITURRINO (R.P.), LUCAS (F.A.), ORGAZ (A.), QUINTANA (J.L.). Estudio comparativo de la eficiencia de un probótico y de vacunas anti-K99 y anti-A14 para el control de la diarrea en terneros en Brasil. *Revue Élev. Méd. vét. Pays trop.*, 1995, **48** (3) : 239-243.

Un total de 99 vacas preñadas se dividió en ocho grupos, sometidos a los siguientes tratamientos : grupo I (n = 29) vacas no vacunadas cuyos terneros no recibieron probiótico y fueron utilizados como controles ; grupo II (n = 10) vacas vacunadas cuyos terneros no recibieron probiótico ; grupos III, IV y V (n = 10 en cada caso) vacas vacunadas cuyos terneros recibieron probiótico durante 5, 15 y 30 días respectivamente ; grupos VI, VII y VIII (n = 10 en cada caso) vacas no vacunadas cuyos terneros recibieron probiótico durante 5, 15 y 30 días respectivamente. Cada animal en los grupos vacunados recibió dos dosis subcutáneas de 5,0 ml de vacuna conteniendo cilios de K99 y de A14 de *Escherichia coli.* El probiótico conteniendo *Lactobacillus acidophilus* a dosis de 2.0×10^8 células vivas en 250 ml de leche se administró vía oral. Todos los animales se siguieron clínica y bacteriológicamente, determinándose los títulos de anticuerpos anti K99 y anti A14 en suero y calostro. El peso promedio de los terneros se obtuvo al nacimiento y a los 30 días de edad. Los resultados demuestran que una combinación de la vacuna con el probiótico administrada durante 15 y 30 días es el tratamiento más eficaz para el control de la diarrea.

Palabras clave : Bovino - Ternero - Diarrea - *Escherichia coli* - Probiótico - Vacuna - Control des enfermedades - Brasil.